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TITLE: Evaluation of the G-quadruplex Binding Drug Telomestatin as an Inhibitor of c-myb in Chronic Myelogenous Leukemia

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14. ABSTRACT

Concept: We propose to investigate MYB as a potential molecular target of telomestatin in CML, and we will seek to answer two major questions about telomestatin in CML. How does telomestatin prevent MYB expression in CML, and is telomestatin active against CML in vivo? We hypothesize that the anti-leukemia activity of telomestatin in CML is in part due to inhibition of MYB expression by the binding of telomestatin to a G-quadruplex in the MYB promoter. Aims: The specific objectives of this proposal are: 1) to evaluate the ability and mechanism of telomestatin to selectively suppress c-MYB expression in K562 and K562R CML cells in culture; and 2) to investigate the anti-leukemia activity of telomestatin in murine K562 and K562R xenografts. In aim 1, we will treat imatinib sensitive and resistant CML cells with telomestatin alone or in combination with imatinib and measure the expression of C-MYB, housekeeping control genes, a panel of other genes that we have identified as containing potential G-quadruplex forming units in their promoters, and telomerase (hTERT) expression. We will show that telomestatin can prevent transcription factor binding to the MYB promoter in solution by EMSAs and footprinting, and in CML cells by ChIP assays. In aim 2, we will form K562 and K562R xenografts in immunodeficient (scid) mice, administer telomestatin to these mice by tail vein injection, and measure the activity of telomestatin on the growth of these CML xenografts. To determine the in vivo mechanism of action of telomestatin, tumors will be harvested and analyzed by standard histopathology, immunohistochemistry for MYB, and analysis of telomerase activity.

15. SUBJECT TERMS
GUANINE QUADRUPLEX, C-MYB, TRANSCRIPTION, PROMOTER, TRANSCRIPTION FACTORS, TELOMESTATIN, MURINE XENOGRAFTS, TELOMERASE, TELOMERE

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Introduction

Telomestatin is a natural product that can bind to G-quadruplex DNA, and telomestatin was reported to possess anti-leukemia activity in CML cells. The project funded by the CMLRP was to explore the activity and potential mechanism(s) of action of telomestatin in CML. Because we had recently discovered a region of the c-myb capable of forming a G-quadruplex, and because c-myb plays an important role in the malignant phenotype of CML, we hypothesized that the anti-leukemia activity of telomestatin in CML is in part due to inhibition of MYB expression by the binding of telomestatin to a G-quadruplex in the MYB promoter.

The specific objectives of this proposal, as written in the original proposal, were:

1. to evaluate the ability and mechanism of telomestatin to selectively suppress c-MYB expression in K562 and K562R CML cells in culture;
2. to investigate the anti-leukemia activity of telomestatin in murine K562 and K562R xenografts.

In aim 1, we proposed to treat K562 with telomestatin, using telomestatin that was provided to a co-investigator, Dr. Hurley, by Dr. Shin-Ya from the University of Tokyo. We proposed to measure cell proliferation by MTS assay to determine the IC50, and then measure the expression of MYB compared to housekeeping control genes (β-actin and GAPDH) by real time PCR. We proposed to evaluate selectivity for MYB against a panel of other cancer-related genes identified in the Hurley and Ebbinghaus laboratories as containing potential G-quadruplex forming units in their promoters. These studies would determine whether and how telomestatin prevents MYB expression in CML cells, and we reasoned that even if these studies did not demonstrate MYB inhibition by telomestatin, useful information on the mechanism and activity of telomestatin in CML would be gained. Based on the work *in vitro,* we proposed to evaluate the anti-leukemia activity of telomestatin in a *scid* mouse models of CML.

Body

MYB amplification or overexpression in leukemias is an important part of the molecular signature of CML. MYB is differentially overexpressed by more than 6-fold in CML in chronic phase (CML-CP) and more than 10-fold in CML blast crisis (CML-BC). Functions of potential significance to hematopoietic cell transformation might relate to MYB's ability to regulate hematopoietic cell proliferation, MYB's effect on important cell cycle genes like c-MYC, or MYB's role in regulating hematopoietic cell differentiation.

A polypurine tract (PPT) containing multiple GGA repeats is an important promoter element in the control of c-myb transcription. GGA repeats have been shown to form unusual DNA structures related to guanine (G) quadruplexes at physiological potassium concentrations. G quadruplexes are emerging as potential therapeutic targets for the treatment of cancer. We had preliminary evidence that the PPT might be able to fold into an intramolecular quadruplex within cells, specifically a structure called a tetrad:heptad (**Figure 1**), composed of a typical guanine tetrad stacked on a guanine-adenine heptad

containing 4 guanines and 3 adenines. Quadruplex formation in the c-myb promoter could be stabilized by the addition of DNA interactive compounds, such as telomestatin, to suppress c-myb transcription.

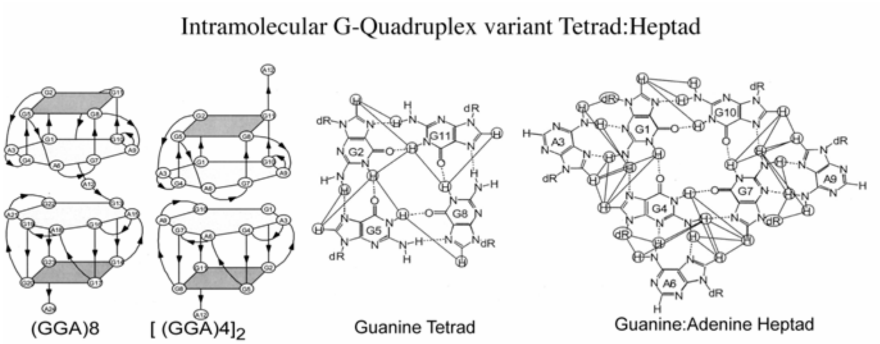

Figure 1. Tetrad:heptad DNA structures formed by oligos with four or eight GGA repeats.

Charaterization of Secondary Structure Formation by the c-myb GGA Repeats

Our studies of G-quadruplex formation by the c-myb promoter present some of the most compelling evidence to date for the relevance of G-quadruplex structures in the control of gene expression in living cells. Our first manuscript resulting from this work was submitted to *Nucleic Acids Research* on May 31, 2007. In brief, chemical footprinting, circular dichroism, and RNA and DNA polymerase arrest assays on oligonucleotides representing the GGA repeat region of the c-myb promoter demonstrate that the element is able to form tetrad:heptad:heptad:tetrad (T:H:H:T) G-quadruplex structures by stacking two tetrad:heptad G-quadruplexes formed by two of the three $(GGA)_4$ repeats. Deletion of one or two $(GGA)_4$ motifs increases c-myb promoter activity, and the relative promoter activity is inversely correlated with the stability of the G-quadruplex formed in the remaining $(GGA)_4$ motifs. Complete deletion of the c-myb GGA repeat region abolishes c-myb promoter activity, indicating dual roles of the c-myb GGA repeat element as both a transcriptional repressor and an activator. Furthermore, we demonstrated that Myc Associated Zinc finger (MAZ) represses c-myb promoter activity and binds to the c-myb T:H:H:T G-quadruplexes. Our findings show that the T:H:H:T G-quadruplex forming region in the c-myb promoter is a critical *cis* acting element and may repress c-myb promoter activity through MAZ interaction with G-quadruplexes in the c-myb promoter. Please refer to the attached manuscript for the experimental details and a full discussion of the results obtained for this important paper.

Evaluation of Telomestatin on MYB Expression in K-562 Cells

We evaluated the ability of telomestatin to suppress c-myb expression in K-562 cells. Cells were treated with increasing concentrations of telomestatin over a range of times, as shown in Figure 2. c-myb mRNA was measured by real time PCR, and normalized with B-actin and GAPDH. We found no change on c-myb expression due to telomestatin treatment at up to 96 hours, as shown in Figure 2, and we only observed a 40% decrease

in c-myb mRNA levels after 7 days exposure to 10 uM telomestatin, conditions which appeared generally toxic to the cells.

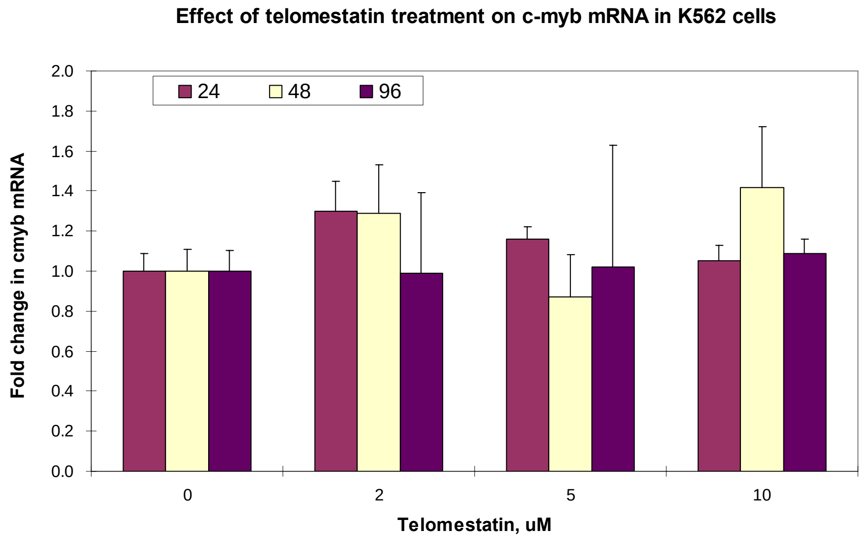

Figure 2. Telomestatin treatment of K-562 cells did not reduce c-myb expression.

From these data, reproduced in multiple experiments, we concluded that c-myb was probably not a molecular target for telomestatin in its anti-leukemic effects on K-562 cells.

The discovery of a G-quadruplex forming region of the hTERT promoter.
Telomestatin was previously reported to induce telomere shortening and inhibit telomerase activity in TRAP assays (Tauchi T. et.al., *Oncogene.* 2003 Aug 14;22(34):5338-47). To verify that we were using a "bioactive" preparation of telomestatin, we performed TRAP assays with K-562 extracts treated with telomestatin, and our results were generally in agreement with the published data, showing >80% inhibition of telomerase activity. However, it is important to note that the TRAP assay does NOT truly evaluate the effect of a compound on the endogenous telomere of the treated cell; rather, in this context, it is a bioassay for the level of functional telomer*ase* enzyme in the cell extracts being tested. Furthermore, in the Tauchi paper describing the effects of telomestatin in CML cells, the authors reported a decrease in hTERT mRNA levels—an observation that we also reproduced. From these studies, we were motivated to re-examine the concept of the telomere as the molecular target of telomestatin. Rather, we hypothesized that the data from these studies in our lab and previously published studies can be re-interpreted to indicate that hTERT, the gene producing the telomerase enzyme, is the molecular target of telomestatin.

We examined the hTERT promoter and found that the critical cis-acting region that contain or overlap with essential Sp1 binding sites contained runs of guanines in motifs that were potentially capable of forming one or more intramolecular guanine quadruplexes (Figure 3). Using synthetic oligomers, we used footprinting and circular

dichroism to show that the hTERT promoter contains sequences capable of G-quadruplex formation. These data lead us to the hypothesis that G-quadruplex formation in the promoter region of the hTERT gene

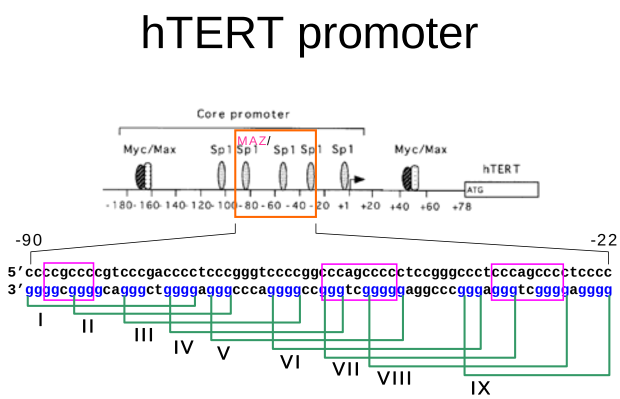

Figure 3. Potential G-quadruplex forming sequences in the hTERT promoter are found in the critical cis-acting element of the core hTERT promoter.

We used a cell-free drug-screening assay, the DNA polymerase arrest assay, to show that telomestatin, as well as the cationic porphyrin TmPyP4, but not its positional isomer, TmPyP2, could interact with the G-quadruplex formed by the hTERT promoter.

The biological significance of these observations is emerging. It is clear that G-quadruplex interactive compounds can decrease hTERT expression by leading a decrease in the hTERT mRNA levels. In our studies, we confirm the observations of Tauchi, et al, by showing that telomestatin can decrease hTERT mRNA levels, and we have extended these observations to include the cationic porphyrin G-quadruplex binding agents. It is also notable that a decrease in hTERT mRNA levels was also reported after treatment with the G-quadruplex interactive compound 12459 by Gomez, et al (*Nucleic Acids Res*. 2004 Jan 16;32(1):371-9), and shown in this paper to result from alternate mRNA splicing. Regardless of mechanism, three independent groups (ours and two others), using different G-quadruplex binding compounds and different cell lines, have now shown that G-quadruplex interactive compounds can suppress hTERT mRNA levels.

To determine whether the decrease in endogenous hTERT mRNA levels is due to a decrease in hTERT transcription by an interaction of the drug with the hTERT promoter, we constructed a cell-line called "293 hTERT P330-luc," containing the 330 bp core hTERT promoter sequence driving the expression of luciferase, integrated into the genome of 293 cells in single copy by using a homologous recombination strategy. We showed that telomerase (not shown) and TmPyP4 (**Figure 4**) significantly decrease hTERT promoter activity in 293 cells

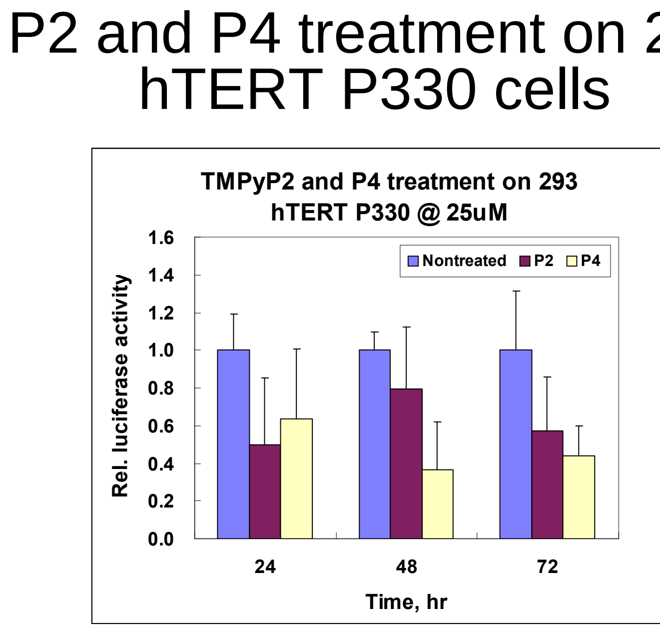

Figure 4. The cationic porphyrin and G-quadruplex binding drug, TmPyP4 leads to a significant reduction in hTERT driven luciferase activity. A smaller effect is also seen with the positional isomer, TmPyP2.

In follow-up, we have shown that hTERT expression falls in a concentration dependent manner in response to treatment with TmPyP4, and at the 100uM concentrations previously required to suppress c-myc expression, the fall in hTERT expression occurs within hours and precedes the fall in c-myc mRNA.

In our work on the c-myb promoter, we showed that the transcription factor MAZ can bind to the G-quadruplex and may be a negative regulator of c-myb expression by binding to the c-myb G-quadruplex (see attached manuscript). Since MAZ is also known to bind in the hTERT promoter in an area that overlaps with the G-quadruplex forming elements (see Figure 3), we speculated that MAZ might be involved in downregulating hTERT expression by recognition of and binding to the G-quadruplex formed in the hTERT promoter. We showed that the forced overexpression of MAZ in the 293 hTERT P330-luc cells markedly decreases hTERT expression, and we have very recently shown by EMSA that MAZ protein can bind specifically to the G-quadruplex conformation of the hTERT promoter.

In conclusion, we propose that the hTERT promoter is a molecular target for telomestatin and other G-quadruplex binding drugs. While the c-myb promoter does not appear to be a molecular target of telomestatin in the treatment of K-562 cells, we have shown that myb regulation very likely involves the formation of an unusual G-quadruplex structure, a tetrad:heptad:heptad:tetrad, and the interaction of a transcription factor, MAZ, with double-stranded and G-quadruplex conformation of the promoter.

Key Research Accomplishments

- Biochemical characterization of the G-quadruplex formed by the c-myb promoter, and submission of manuscript describing these results to *Nucleic Acids Research.*.
- Drug testing of telomestatin on K-562 cells, and analysis of target gene expression.
- Discovery of the G-quadruplex forming region of the hTERT promoter.
- Elucidation of the mechanism of action of telomestatin by demonstrating that G-quadruplex ligands suppress hTERT expression by a direct interaction with the hTERT promoter.

Reportable Outcomes (reprints, presentations, patents, etc.)

Submitted Manuscript

SunMi Lee Palumbo, Regan M. Memmott, Diana J. Uribe, Yulia Krotova-Khan, Laurence H. Hurley, and Scot W. Ebbinghaus, A novel G-quadruplex forming GGA repeat region in the c-myb promoter is a critical regulator of promoter activity, submitted to *Nucleic Acids Research* on May 31, 2007.

Conclusions

In conclusion, we propose that the hTERT promoter is a molecular target for telomestatin and other G-quadruplex binding drugs. While the c-myb promoter does not appear to be a molecular target of telomestatin in the treatment of K-562 CML cells, we have shown that myb regulation very likely involves the formation of an unusual G-quadruplex structure, a tetrad:heptad:heptad:tetrad, and the interaction of a transcription factor, MAZ, with double-stranded and G-quadruplex conformation of the promoter.

References cited

None